with no antigen added were treated as background. The mixture of antigen, biotinylated antibody and streptavidin Alexa 546 was allowed to incubate in the array in the channels for 15 min. Standard curves were obtained for each biomarker which are seen in FIG. **26** A-E. Data points for each curve represent the average intensities of two replicate samples (and hence eight different spots) with a coefficient of variation of approximately 15% for all protein biomarker curves. The standard curves for all four biomarkers appear to be linear in the clinically relevant ranges. Sensitive and linear response is observed for all the four biomarkers including Her-2 and MMP-2 in the concentration range selected. T his therefore confirms that Alexa 546 works well as the molecular reporter for our multiplexed assay on microarray channels.

Multiplexed Assay on the Flow Channels

Microarray flow channels were used to simultaneously detect multiple biomarkers one single sample. In this experiment, eight identical slides were printed with capture antibodies to the four protein biomarkers printed in quadruplicate shown as columns in FIG. **27**. O=OPN, C=CA 15-3, H=Her-2, and M=MMP-2. Four arrays were incubated with a mixture of all four but one biomarker (A) and the other four slides were incubated with only one antigen (B). Fluorescence was observed on the spots where the corresponding antigens were added. Some background signal is observed from the spots where no corresponding antigen was added to the mixture. Since human serum was used as the medium of dilution, the non specific binding of the serum proteins to the capture antibody spots as well as the low, normal circulating levels of the biomarkers generate this background signal from the spots even the recombinant antigen was not added. This data therefore demonstrates specific and sensitive detection of the four biomarkers in a multiplex format on the microarray channel device.

The new device platform was designed to measure multiple biomarkers and to produce rapid and reliable results in less than 15 minutes. Multiplexed assay with four different interaction times were therefore performed. A set of eight arrays were printed with all four capture antibodies. Four of these arrays were incubated with a mixture of all antigens in the high concentrations as observed in cancer, while the other set of four arrays was incubated with a mixture of all four antigens in the lower concentrations as observed in normal sera. The concentrations of antigens used to represent metastatic cancer were 30 ng/ml for Her-2, 850 ng/ml for MMP-2, 150 U/ml for CA 15-3 and 875 ng/ml for Osteopontin and the concentrations of antigens used to represent normal sera were 8 ng/ml for Her-2, 600 ng/ml for MMP-2, 15 U/ml for CA 15-3 and 440 ng/ml for Osteopontin. One array from each of the sets was incubated for 7 min, 15 min, 30 min and 60 min respectively. The median fluorescence intensities of the four biomarker spots were measured and are displayed in FIG. **28** (A-D). The fluorescent signal increases were observed with a longer incubation time for all the biomarkers. However, the difference between a 30 min incubation and 60 min incubation is not as drastic as the difference between a 7 min and 15 min incubation. This indicates that the assay reaches equilibrium somewhere between 30 and 60 minutes. Differential signal between the high concentrations (representing metastatic disease) and low concentrations (representing normal sera) is tabulated in FIG. **28** (E). The ratio of this differential signal shows that the ratio for the 7 minute incubation is below 2.0 but above 1.5 for Her-2 and MMP-2, however this ratio is very high for CA 15-3 and MMP-2. This implies that only CA 15-3 and Osteopontin are appropriate for a 7 min diagnostic test. However, all biomarkers have a ratio greater than 2 for a 15 min assay making this the best compromise between assay speed and sensitivity

Flow Channel Patient Serum Immunoassays

To test the power of the flow channels to resolve signals from cancer versus non-cancer samples accurately, breast cancer patient serum samples were incubated on antibody arrays. Since the serum samples were limited, the assay could not be performed with duplicates, similar to the protein microarrays. Therefore, this assay was designed in two parts. First, sera from 10 metastatic breast cancer patients and 10 control subjects was pooled to obtain a total of 800 μl of metastatic breast cancer sample and 800 μl of control sample. This assay eliminated the patient to patient variation, but enabled the measurement of technical variations across various channels and slides. An 80 μl aliquot of this pooled sample (mixed with biotinylated detector antibody cocktail and streptavidin Alexa 546) was drawn across 10 replicate arrays for 15 min. The resulting fluorescent intensities obtained for the four biomarkers are plotted in FIG. **29** (A). Significant differences between metastatic and control populations are observed for all four biomarkers with minimal technical variations (10%).

In the second part of this study, 80 μl of patient sera from 6 metastatic breast cancer patients and 6 control subjects was mixed with biotinylated detector antibody cocktail and streptavidin Alexa 546 reporter and incubated on the arrays for 15 minutes. The fluorescent signals from the four biomarkers were quantified and the median intensities were computed, which is shown in FIG. **29**(B). A t-test was performed on the two sample sets (metastatic and control) for all four biomarkers and a p-value was generated to estimate the resolving power of the system for accurately identifying cancer vs. non-cancer samples. The table of these p-values is listed in FIG. **29** (C). We observe that there is a significant difference between the signals obtained for metastatic and control samples for CA 15-3 (C) and Osteopontin (D). This difference reduced for Her-2 (A) and MMP-2 (B), however, p value table indicates that the channel assay is sensitively ($p<0.05$) able to distinguish between metastatic and control populations for all four.

Optical Reader

A benchtop was built of a rugged, portable fluorescence imager, whose components include a miniature, megapixel CCD camera and a high power xenon arc lamp white light generator. Arrays were exposed to bandpass-filtered excitation light from the xenon source. The resulting emitted light was bandpass-filtered and collected by the CCD camera. The fluorescence images are exported to Scanarray™ for subsequent analysis, where background is calculated by taking into account the autofluorescence inherent to the glass slide and the non-specific binding of fluorescence probe material in the area surrounding the target spots on the array. FIG. **30** shows images of microarray channels used to obtain standard curves for all four biomarkers as captured by the CCD based imaging system. Channels shown in FIG. **30**(A) were incubated with Her-2 at concentrations ranging from 6.25 ng/ml (right) to 100 ng/ml (left). Channels shown in Panel B were incubated with MMP-2 with a concentration range of 62.5 ng/ml (right) to 1000 ng/ml (left). Channels shown in Panel C were incubated with CA 15-3 at concentrations ranging from 9.4 U/ml (right)-150 U/ml (left) and those shown in Panel D were incubated with Osteopontin at concentrations from 94 ng/ml (right) to 1500 ng/ml (left). Human serum was used as the diluting medium in these assays. The results show increased fluorescence intensity with increased protein concentration.

The fluorescence from these spots was quantified using the Scanarray software and plotted as a function of antigen con-